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Project Title: Effect of intracellular delivery of thioredoxin-1 on a cell line model of Parkinson's disease

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Summary (250 words max single spaced):

Parkinson Disease (PD) is a common neurodegenerative disease that affects 1-2% of the people over the age of 65. The projected prevalence of PD is estimated to double by 2040. Progressive loss of dopaminergic neurons in substantia nigra is the main pathophysiological feature of PD, resulting in motor and non-motor symptoms. Current pharmacological treatments aim at temporarily alleviating the motor symptoms; however a neuroprotective cure remain to be identified.

PD is identified by oxidative stress mediated cell death, and therefore antioxidant molecules are a potential therapy to promote cell survival. The thioredoxin system represents a main candidate for therapeutic purposes as the level of endogenous thioredoxin is substantially decreased in the PD brain. In this project I aimed to investigate the therapeutic potential of a modified thioredoxin protein that can traverse the cell membrane. An in vitro model of PD was used in this study and we investigated the potential therapeutic effect of our intracellular thioredoxin delivery method. Although our studies showed that protein transduction technology employed in these experiments enhanced cell viability in this model, the underlying mechanism for this modest increased protection remains to be elucidated.

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Introduction and Background

Parkinson disease (PD) is the second most common neurodegenerative disorder with a prevalence of 65,000 in Canada, affecting roughly 1-2% of the population over the age of 65¹. PD is characterized by the progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNc) resulting in dopamine deficiency in the putamen of the dorsolateral striatum². Clinical manifestations include motor symptoms such as bradykinesia, rigidity, tremor, and postural instability. The current treatments of PD, including levodopa, dopamine agonists, and monoamine oxidase inhibitors, mainly improve motor symptoms, but do not prevent or delay the degenerative process, often losing their effectiveness over time. The lack of a neuroprotective treatment indicates the need for a better understanding of the underlying mechanisms of PD pathogenesis and better therapeutic targets.

Oxidative damage to cellular macromolecules is the common cause for neuronal death in PD and other neurodegenerative diseases. This is attributed to the malfunction of mitochondria and enhanced generation of Reactive Oxygen Species (ROS). In familial early-onset forms of PD, there is a loss of function of genes such as DJ-1, PINK1, and Parkin which function in anti-oxidant pathways, regulation of mitochondrial health, and clearance of damaged mitochondria. Moreover, toxins which disrupt complex I in the electron transport chain of mitochondria, such as 1-methyl-4-phenyl tetrahydropyridine (MPTP) and rotenone, produce Parkinson-like symptoms and brain pathology in mammals^{3,4}. While normal levels of ROS play a vital role in cell signalling, excessive ROS levels can result in the oxidation of key molecules, including those involved in the regulation of cell death. Therefore, scavenging ROS by antioxidant molecules in oxidative stress conditions like PD is an important area of study.

Endogenous antioxidant molecules in neural cells include small thiol peptides and proteins that are responsible for reducing ROS and oxidized proteins. Thioredoxin (Trx) is one of the major antioxidants involved in regulation of ROS levels and maintenance of the cellular reducing/oxidizing balance. The thiol groups at the active site of Trx can quickly donate their available protons to key proteins that have been inactivated due to oxidation by ROS. In turn, the oxidized Trx is reduced by thioredoxin reductase (TrxR), which uses NADPH as a source of electrons. Trx and TrxR genes are important players in the cell's response to oxidative stress; increased levels of ROS results in translocation of Nrf-2 (nuclear factor erythroid-2 related factor) into the nucleus, which then binds to the antioxidant response element (ARE) and induces transcription of Trx, TrxR and with other protective enzymes. Along with its anti-oxidative effects, Trx regulates important transcription factors, exhibiting anti-apoptotic and anti-inflammatory effects⁵, and acting as a neurotrophic co-factor⁶.

Evidence of the protective role of Trx in PD has been suggested in human autopsies; Trx is highly expressed in normal SNc⁷, but in post-mortem PD brains, it has been shown that Trx levels are decreased⁸. Treatment of SH-SY5Y cells with neurotoxins which induce mitochondrial dysfunction and/or oxidative stress, such as MPP, rotenone and 6-OHDA have been used as experimental models of PD. Indeed, in SH-SY5Y cells treated with 6-OHDA, a decrease in Trx levels has been reported corresponding to an increase in oxidative stress and cell death⁸. Trx therapeutic properties have also been shown in SH-SY5Y cells treated with MPP, where application of exogenous human recombinant Trx exhibited an anti-apoptotic effect⁹. Due to the

neuroprotective role of thioredoxin in dopaminergic neurons, it has potential to be a novel protein therapy in Parkinson's disease.

While Trx is a small protein (12kDa) it cannot cross the blood brain barrier, making protein therapy a difficult approach⁵. We therefore hypothesized that an intracellular delivery method may improve the therapeutic application of Trx. We generated a modified human Trx-1, subsequently named intracellular thioredoxin (I-Trx), which can quickly enter cells. Application of I-Trx in the central nervous system after a peripheral injection in rodents enhances Trx concentration in the brain and spinal cord. In this proposal we aimed to investigate the effect of intracellular delivery of Trx on neuronal preservation in a cellular model of PD.

Materials and Methods

Production of recombinant thioredoxin protein

Recombinant thioredoxin protein constructs were routinely produced by the Eftekharpour lab following established protocol. Briefly, cDNA was constructed by Integrated DNA Technology (USA) and cloned into pET-28a(+) vector. Recombinant BL21 bacteria were grown in Luria-Bertani (LB) media containing 50µg/ml kanamycin at 37°C, and protein production was induced for 6 hrs using 0.5µM isopropyl-B-D- thiogalactopyranoside (IPTG). Next, bacteria were pelleted down and lysed by French Press. Resulting lysate was incubated Ni-NTA resin (QIAGEN) and run through resin specific binding column. Recombinant thioredoxin protein was eluted from the column after repeated washes with imidazole. Eluent was subsequently run through a desalting column and endotoxin removal column. Finally, proteins were dissolved in PBS, sterile filtered, and stored in -80°C after the measurement of protein concentration via Pierce BCA Protein Assay Kit. Six constructs were used in this project as seen in Figure . Insulin reduction assay was used to confirm the reducing capability of each protein.

Cell culture and treatment

Human SH-SY5Y neuroblastoma cell line was a kind gift from Dr. Jun-Feng Want, University of Manitoba. The SH-SY5Y cell line was grown in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/L) supplemented with 4mM glutamine, 1mM sodium pyruvate, 2mM penicillin/streptomycin and 10% heat inactivated Fetal Bovine Serum, and incubated in 5% CO₂ at 37°C. For differentiation of SH-SY5Y, growth media was replaced with 1% FBS media plus 10µM retinoic acid for 7 days, with a media change on day 4. For treatment with N-Methyl-4-phenylpyridinium iodide (MPP⁺) (Sigma Aldrich), a 30 mM stock solution was freshly prepared for each experiment, and diluted to desired concentration in 1% FBS media. Recombinant protein treatments were diluted in MPP⁺ media according to experimental conditions and added to cells at ~80% confluence.

Immunocytochemistry

Cells were cultured on glass coverslips and treated according to experimental conditions. After the 24 hours, cells were washed once with PBS, then fixed with 3% paraformaldehyde in PBS. Coverslips were blocked and permeabilized in 0.3% TritonX-100, 1% BSA, 5% milk diluted in PBS, then incubated with primary antibody overnight in 4°C. Coverslips were then washed three times with PBS, and incubated with secondary anti-body. Lastly, coverslips were stained with DAPI (1:10000) to visualize the nuclei, and then mounted onto glass microscope slides. Fluorescence microscopy was performed using a LSM710 Zeiss confocal microscope.

The following antibodies were used at their specified dilutions: chicken vimentin (1:1000) obtained from Chemicon; mouse β -tubulin III (1:300) from promega; cytochrome c (1:100) from Santa Cruz. AlexaFluor secondary antibody (1:1000) was obtained from Invitrogen.

Cell viability

Cell viability was assessed using MTT assay and Cell Counting Kit-8 (CCK-8 kit) (Dojindo Molecular Technologies, Japan). SH-SY5Y cells were seeded in 96 well plates at a density of 10,000-15,000 cells/well in 100 μ l of growth media. Cells were incubated for 24-48 hours at 37°C to allow for adhesion, then differentiated and/or treated according to the experimental conditions. After the appropriate treatment period, 10 μ l of MTT or CCK-8 reagent was added to each well and incubated for 4 hrs. For the MTT assay, media was removed and crystals were dissolved in DMSO. The absorbance was then measured at 570nm with background correction at 690nm using Synergy H1 Hybrid reader (BioTek Instruments, USA). For the CCK assay, the absorbance was measured directly after incubation at 450nm with background correction at 650nm.

LDH assay

Membrane integrity was assessed using LDH cytotoxicity detection kit (Roche Life Science, USA). SH-SY5Y cells were grown and treated in 96 well plates as above. After the treatment period, media was removed from the cells. Catalyst/dye solution was mixed with the media in a 96 well half area plate and incubated for 30 minutes at room temperature. Stop solution was added and the absorbance was read at 490nm with background correction at 650nm with the plate reader.

Western Blot

Treated cells were scraped, collected by centrifugation at 5000rpm for 5 min at 4°C, washed once with PBS, and re-centrifuged before being stored as a cell pellets in the -80°C freezer. Cell pellets were lysed in NP-40 lysis buffer (50mM Tris HCL pH 8, 150 mM NaCl, 5mM EDTA, 1% NP-40, 1X protease and phosphatase inhibitor) by sonication in ice (3 pulses, 5 sec on, 15 sec off), then centrifuged at 12,000 rpm for 15 min at 4°C to collect the supernatant. The protein concentration of the cell extracts were measured using the Pierce BCA Protein Assay Kit, then diluted to equal protein concentrations in loading buffer and boiled for 10 minutes.

Samples were resolved by SDS-PAGE and transferred to PVDF membrane following routine Western protocol. Membranes were blocked for 1 hr with 5% skim milk in Tris-buffered saline and 0.2% Tween 20 (TBST), and incubated with primary antibody diluted in 1% skim milk overnight at 4°C. After three washes with TBST, membranes were probed with HRP-conjugated secondary antibody diluted in 1% skim milk for 1 hr at room temperature. Membranes were again washed, and imaged using ECL prime detection reagent (GE Healthcare Life Sciences). As a loading control, membranes were incubated with HRP-Actin antibody for 3 hrs, washed, and imaged. Measurement of band intensity was done using AlphaEaseFC (version 6.0.0, Alpha Innotech) and analyzed as fold change from control.

The following antibodies were used at their specified dilutions: rabbit LC3 (1:2500) obtained from Sigma, USA; rabbit cleaved Caspase-3 (1:000), rabbit PARP-1 (1:1000), and rabbit IgG HRP-conjugated secondary antibody obtained from Cell signalling Technologies, USA; mouse SQSTM1 (p62, 1:1000) and HRP-conjugated actin (1:4000) were obtained from Santa Cruz Biotechnologies, USA; mouse IgG HRP-conjugated secondary antibody was obtained from Promega.

Glutathione assay

Glutathione assay reagent preparation and protocol were based off of Rahman et al.¹⁰. Cells were cultured and treated with MPP and recombinant protein as previously described, and cell pellets were stored in -80°C. Cell pellets were lysed by sonication in extraction buffer and two freeze thaw cycles before supernatant was collected. Protein concentrations were measured using the Pierce BCA Protein Assay Kit. For measurement of reduced glutathione levels (GSH), refer to Rahman et al.¹⁰. Briefly, samples were added to a 96 well plate and mixed with 5,5'-dithiobis(2-nitrobenzoid acid) (DTNB) and glutathione reductase solution, then with β -NADPH. The plate was read with absorbance at 412 nm in a plate reader and compared to a GSH standard curve.

Measurement of ROS

Cells were cultured on glass coverslips and treated according to experimental conditions. After the 6 hours, cells were incubated in 2.5 μ M CellROX Deep Red (Thermo Fisher) reagent for 30 minutes at 37°C. Cells were washed once with PBS, then fixed with 3% paraformaldehyde in PBS. Coverslips were stained with DAPI (1:10000), then mounted onto glass microscope slides. Fluorescence microscopy was performed using a LSM710 Zeiss confocal microscope and mean fluorescence intensity per cell was measured using ImageJ.

Statistical Analysis

The statistical analysis was carried out by one way analysis of variance (ANOVA) to compare multiple groups followed by Tukey's post-hoc test. All values were expressed as mean \pm S.E.M. P-values less than 0.05 ($p < 0.05$) were considered as significant.

Results

Effect of exogenous recombinant thioredoxin on cell viability and cell death in MPP treated SH-SY5Y cells

A study by Andoh et al. showed that administration of exogenous Trx enhances cell protection mediated through inhibition of apoptosis in SH-SY5Y cells treated with 1mM MPP for 24 hrs⁹. We therefore aimed to test the efficacy of our protein constructs on cell viability in this in vitro model of PD (Fig. 2a). A significant increase in cell viability was observed with I-Trx in comparison with E-Trx and MPP only treatment. We also used a mutant version of Trx protein that can also enter the cell but does not have the redox regulating capacity of I-Trx. Interestingly, co-treatment of MPP-treated SH-SY5Y with mutant I-Trx (ml-Trx) also enhanced cell viability. This indicates that Tat-mediated protein transduction into the cells may have triggered a protective mechanism. Complementary cell death assessment using LDH assay was also performed in these experimental conditions (Fig. 2b). LDH release was increased by 1.5 fold with MPP; however there was no significant change in cell death with our protein treatment compared to MPP only treatment.

Cell survival was also visually examined using phase contrast bright field microscopy in cultures of SH-SY5Y cells treated with MPP and recombinant thioredoxin proteins (Fig. 2c). We observed that in comparison with the control cultures, MPP-treated cells had a marked decrease in cell density and showed prominent signs of cellular damage as seen by cell shrinkage and retraction of processes. In contrast, protein transduction using I-Trx (Fig. 2c), E-Trx, and ml-Trx (not shown) all resulted in improved cell morphology, including a notable increase in cell density with well-defined neurite-like processes.

Effect of exogenous recombinant thioredoxin on induction of apoptosis markers in MPP treated SH-SY5Y cells

To further investigate the underlying mechanism in the observed protective effect of thioredoxin proteins on MPP cytotoxicity, MPP-treated SH-SY5Y cells, we hypothesized that protein treatment may affect the balance between apoptosis and autophagy. Apoptosis, also known as programmed cell death, is one of the processes that cells may undergo during stressful conditions. Apoptosis can be mediated through the intrinsic pathway in response to mitochondrial damage or the extrinsic pathway mediated by surface receptor. Both of these pathways result in activation of caspase-3, the executioner of apoptosis. Subsequently, caspase-3 activation can lead to activation of PARP-1, further promoting apoptosis. In our experimental model, no evidence of caspase-3 activation was observed in control cells while a prominent signal indicating the activation of caspase-3 was observed after MPP-treatment (Fig. 3a). Interestingly, a significant decrease in cleaved caspase-3 was observed for all Trx constructs, including the mutant form (ml-Trx). Another marker for activation of apoptosis machinery is the activation of PARP1 in a caspase-3 dependent manner. PARP-1, is an important DNA repair enzyme, however upon its activation by Caspase-3 enhances ATP consumption which results in cell death. Compared to the control group, the MPP treatment groups showed a 3-fold increase in cleaved Parp-1 levels (Fig. 3b). There did not appear to be any difference between MPP only treatment and any of the thioredoxin treatment groups.

Effect of exogenous recombinant thioredoxin on induction of autophagy markers in MPP treated SH-SY5Y cells

Autophagy is a survival mechanism that is induced under oxidative stress conditions. In this process, cells recycle their damaged proteins and organelles by sequestering them into autophagosomes. These organelles and proteins are then degraded through the action of lysosomes, generating energy for cellular maintenance. Prolonged autophagy during stress conditions ultimately results in termination of autophagy and induction of apoptosis. Indeed treatment of SH-SY5Y cells with MPP has been shown to induce autophagy in SH-SY5Y cells¹¹.

We assessed the induction of autophagy by Western Blot in our experimental model (Fig. 4a). Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a major marker of autophagy. Cytoplasmic LC3 (LC3-I) is incorporated onto the membranes of autophagosomes whereby they become lipidated LC3 (LC3-II). This conversion of LC3-I to LC3-II can be resolved by Western Blot as the appearance of a lower molecular weight band. Therefore the ratio between LC3-II to LC3-I can be used to measure rate of autophagy. In our experiments, we saw a strong LC3-II band across all treatment groups including control, and no significant difference in LC3-II/LC3-I ratio. While a low LC3-II level in the control group should be expected, a basal level of autophagy may have been induced in our cells, as serum content was decreased in the growth media from 10% FBS to 1% FBS during the 24 hour treatment period. This may have masked any apparent change in autophagy rate between MPP and Trx construct treatments.

Another important marker for autophagy is p62, a key protein involved in tagging proteins destined for digestion in lysosomes. With an increase in autophagy activity, p62 levels tend to decrease as they become sequestered and degraded in autophagosomes. On the other hand, the accumulation of p62 can mean an interruption of autophagy. Our data showed that very low levels of p62 was observed in control and MPP-treated cells (Fig. 4b); however, there was a marked increase in p62 levels with all our protein treatment groups, with no significant difference between groups. While this increase in p62 may indicate a blockage in autophagy progression, it is also possible that p62 production is being upregulated in response to our exogenous protein treatment. It is unclear whether the induction of p62 is a contributing factor to the increased cell viability seen with our protein treatments. One mechanism by which p62 may contribute to increased cell viability may involve the upregulation of endogenous anti-oxidant proteins; accumulation of p62 level can upregulate Nrf2 function, ultimately inducing ARE gene expression and producing a protective response¹².

Effect of exogenous recombinant thioredoxin on glutathione levels in MPP treated SH-SY5Y cells

Glutathione (GSH) is the most abundant antioxidant in cells which can buffer ROS production. Indeed a decrease in reduced glutathione levels has been found in substantia nigra of PD patients¹³. GSH gene expression is also regulated by the ARE in its promotor region¹²; therefore GSH levels can be used as an indirect assessment of NRF2 activity. In this study we predicted that the protective effect of protein therapy may indirectly induce changes in GSH in our MPP treatment model. Our data shows that MPP treatment does decrease GSH, while co-treatment with N-acetylcysteine (NAC), a precursor for glutathione synthesis, increases GSH. Thioredoxin treatment did not result in improvement in GSH levels; however, I-Trx showed a

further decrease in GSH (Fig. 5). Interestingly control group with I-Trx protein also showed a significant decrease in GSH when compared to control. This may indicate a downregulation of GSH expression related to the ability of our I-Trx protein to enter and accumulate in the cell compartments.

Effect of exogenous recombinant thioredoxin on ROS levels in MPP treated SH-SY5Y cells

Since MPP induces oxidative stress in SH-SY5Y cells, we studied the effect of recombinant thioredoxin on ROS levels through fluorescence staining with CellROX (Fig. 6b). As expected, we see an increase in ROS levels in the MPP treated groups. However, our I-Trx treated cells in both control and MPP groups showed an even greater increase in ROS level. While this is a paradoxical finding to the redox capability of I-Trx, it does correlate with the decrease in GSH content seen with I-Trx treatment after 24 hrs in the results from the glutathione assay (Fig. 5).

Discussion

This project aimed to test recombinant thioredoxin proteins on MPP induced cytotoxicity in SH-SY5Y cells as a potential therapy for PD. Our results showed that while intracellular protein treatment showed some cell protection, the underlying mechanism for this protection could not be identified. This is the first attempt to use intracellular protein delivery for treatment of PD in a cellular in vitro model. Parallel studies in our lab with in vivo application of these proteins have provided promising protective effects when administered into animal models of stroke and spinal cord injury.

SH-SY5Y cells are used as a cell line model for PD due to their dopaminergic phenotype. Studies have shown the effectiveness of thioredoxin in both undifferentiated and differentiated SH-SY5Y cells treated with neurotoxin^{9,14}. However, there are conflicting conclusions on whether differentiation of SH-SY5Y provides a better model of PD. In theory, cells that are more sensitized to the neurotoxin are a better candidate. Lopes et al. has shown that retinoic acid (RA) differentiated cells increase sensitization to neurotoxins¹⁵. This is in contrast to Cheung et al., who shows that differentiation induces survival pathways which decrease their sensitivity¹⁶. More consistent data shows that differentiation of SH-SY5Y cells with RA induces a more dopaminergic neuronal phenotype while also decreasing oncogenic and mitogenic properties^{15,17}. This likely better mimics neurons in vivo.

In our study we used both differentiated and undifferentiated SH-SY5Y cells. We were unable to show a significant change in cell viability and cell death in RA differentiated neurons with MPP and recombinant Trx co-treatment (Fig. S2). We therefore committed to using only undifferentiated SH-SY5Y cells in subsequent experiments. Improved cell viability was shown with our thioredoxin treatment, but there was still significant cell death as shown by LDH release assay. A potential mechanism for the increase in cell viability and decrease in cleaved caspase products seen in our experiments is through the interaction of thioredoxin with ASK1. In

response to oxidative stress, thioredoxin becomes oxidized and dissociates from ASK1, starting a pro-apoptotic signalling cascade¹⁸. Treatment with exogenous thioredoxin may therefore prevent cell death. However, studies have shown that binding with ASK1 requires the two active cysteine residues which are replaced with serine in our ml-Trx proteins; in our experiments, ml-Trx provided similar increase in cell viability as I-Trx detracting from this mechanism of protection.

Another hypothesis is that there is a potential effect of Trx on cell proliferation contributing to increased cell viability. There is mounting evidence that thioredoxin has a role in neuronal proliferation, growth, and differentiation; thioredoxin acts as a neurotrophic cofactor to nerve growth factor (NGF)⁶ and recombinant human Trx1 protein has been shown to increase the proliferation and differentiation of neural stem cells¹⁹. Since our model involves proliferative neuronal cells, this may account for the change in cell morphology and increase in cell number observed with our Trx treatment.

An interesting finding in this study is that our I-Trx protein treatment increased ROS levels and depleted glutathione levels. There is evidence that reductive stress paradoxically induces the production of ROS by directly donating electrons to oxygen, forming hydrogen peroxide. Since I-Trx is capable of easily entering the cell, it can accumulate at high concentrations, producing a reductive stress on the cells. Despite these findings, we were still able to show an increase in cell viability. Therefore, future experiments will be aimed at determining the optimal concentration of I-Trx in this model of PD to maximize protection.

Autophagosomes have been shown to accumulate in PD brains and with in vitro models, as shown by an increase in LC3-II levels with MPP or rotenone treatment^{11,20,21}. While autophagy is an adaptive response to oxidative stress, it may also become pathological. Both Wang et al. and Zhu et al. suggest that inhibition of autophagy in PD models can increase cell survival^{11,20}. In our experiments we observed that our control group exhibited an appreciable level of LC3-II, whereas LC3-II levels should be very low in control cells treated in normal growth conditions. Therefore we were unable to conclude whether autophagy was upregulated with MPP treatment. However, we also showed that our protein treatment induced a significant increase in p62 levels. This was not a thioredoxin dependent effect, as treatment with exogenous red fluorescent protein, which has no enzymatic activity, also induced an increase in p62. This may indicate that exogenous RFP linked proteins are recognized and tagged with p62 in these cells, likely sequestering them into autophagosomes for degradation. Indeed, preliminary immunocytochemistry data with lysosomal membrane associated protein (LAMP-1) staining suggests that I-Trx may be targeted towards the lysosomal compartments in SH-SY5Y cells.

In conclusion, we showed that intracellular delivery of thioredoxin protein enhanced cell viability on a cell line model of PD. However, the exact mechanism of protection remains to be elucidated. Future experiments will focus on maximizing the cytoprotective effect of I-Trx, including further study of the effect of lysosomal degradation on these recombinant proteins. Since mitochondrial dysfunction is a hallmark of PD, the effect of I-Trx treatment on MPP induced mitochondrial fragmentation, change in mitochondrial potential, and change in ATP levels are logical next steps.

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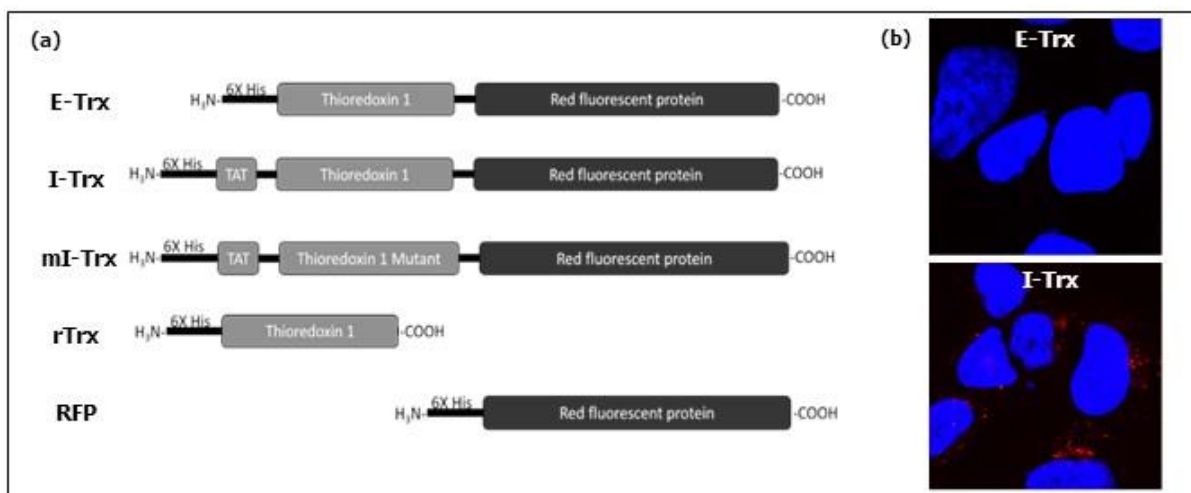


Figure 1: Recombinant thioredoxin constructs.

(a) Each recombinant thioredoxin contains a 6x His tag at the N-terminal end followed by human thioredoxin-1 protein. Red fluorescent protein is tagged at the C-terminal end. To aid protein delivery into cells, Tat sequence is added to the N-terminal end of intracellular thioredoxin (I-Trx) while extracellular thioredoxin (E-Trx) has no TAT sequence. Mutant variants of human thioredoxin lacked cysteine residues required for their redox activity and were constructed with TAT (mI-Trx). Tat tagged thioredoxin-1 (rTrx) and red fluorescent protein (RFP) alone were also constructed. (b) Representative images of intracellular localization of I-Trx at 2 μ M concentration in SH-SY5Y cells.

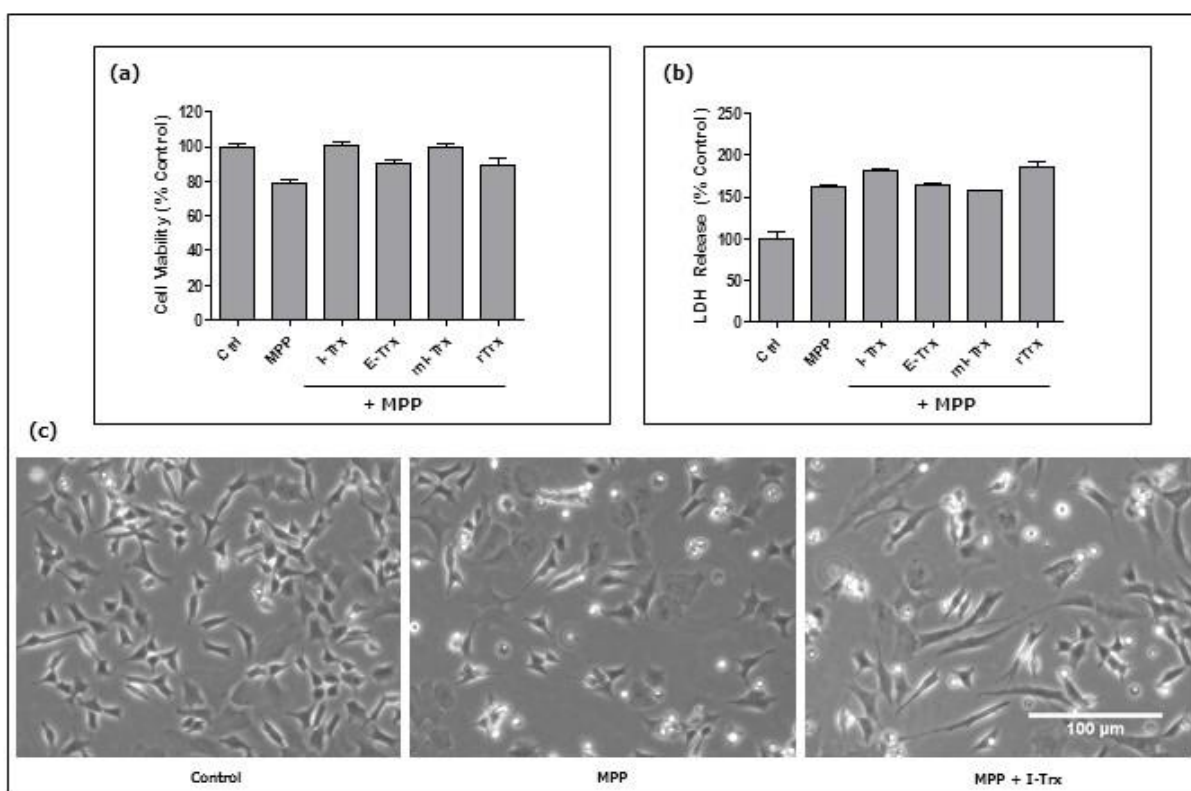


Figure 2: Effect of recombinant thioredoxin on cell viability and cell death in MPP treated SH-SY5Y cells

SH-SY5Y cells were treated with 1mM MPP+ plus 2 μ M of recombinant protein in 1%FBS media for 24 hrs. (a) Cell viability measured using Cell Counting Kit-8 (CCK-8). (b) Cell death measured using LDH assay. In both assays, each treatment group is expressed as percent control, mean \pm S.E.M., $n \geq 3$. (c) Representative images of treated SH-SY5Y cells taken with phase contrast bright field microscopy at 20X magnification.

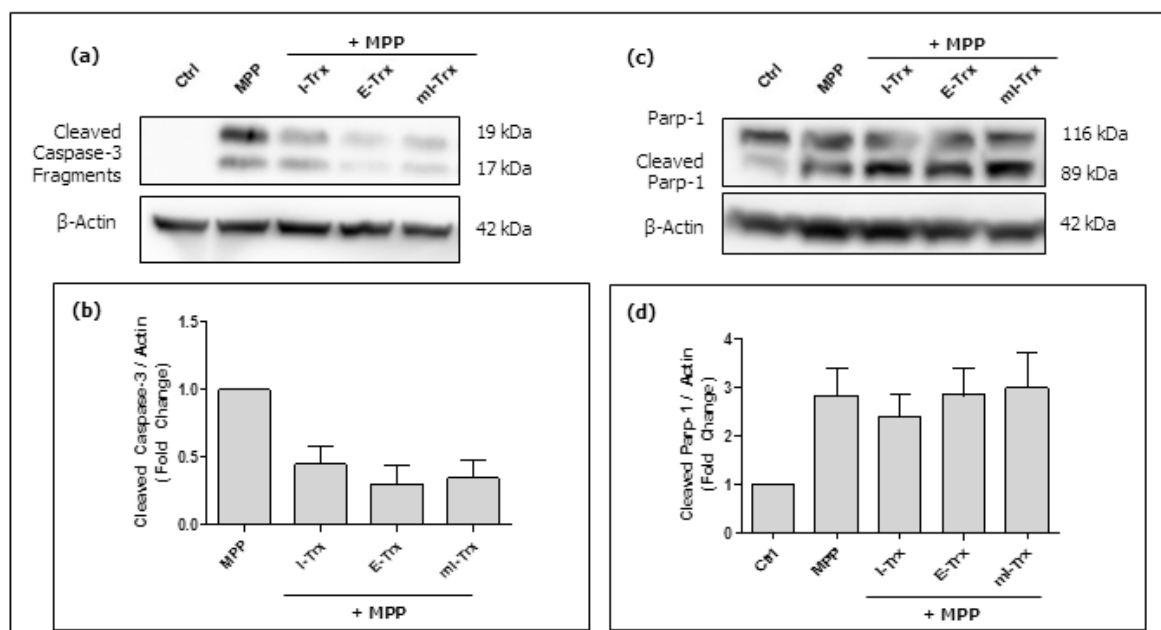


Figure 3: Effect of recombinant thioredoxin on apoptotic markers in MPP⁺ treated SH-SY5Y cells
SH-SY5Y cells treated with 1mM MPP⁺ plus 2 μ M of recombinant protein in 1%FBS media for 24 hrs were assessed for apoptotic markers by Western Blot. **(a)** Representative blot showing cleaved Caspase-3 fragments. **(b)** Quantification of cleaved Caspase-3 fragments / Actin levels. Protein treatments are compared to MPP⁺ only treatment as control. Values are expressed as mean \pm S.E.M., n=4. **(c)** Representative blot showing expression of Parp-1 and cleaved Parp-1. **(d)** Quantification of Cleaved Parp-1 / Actin levels. Values are expressed as mean \pm S.E.M., n=5.

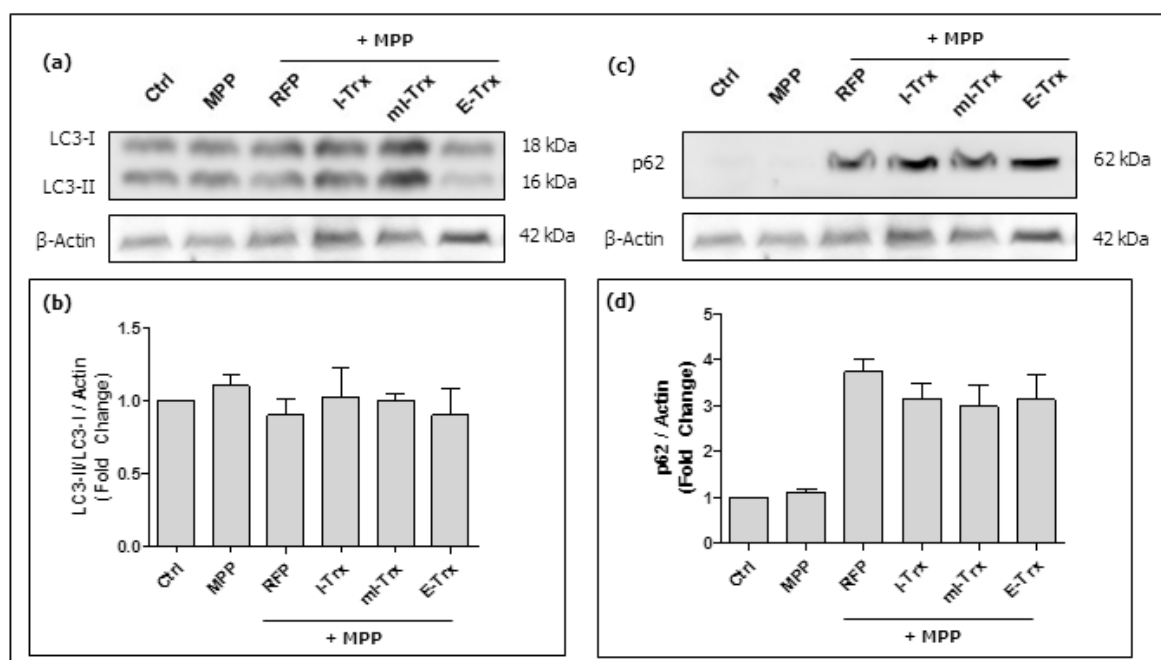


Figure 4: Effect of recombinant thioredoxin on autophagy markers in MPP⁺ treated SH-SY5Y cells
SH-SY5Y cells treated with 1mM MPP⁺ plus 2 μ M of recombinant protein in 1%FBS media for 24 hrs were assessed for autophagy markers by Western Blot. **(a)** Representative blot showing LC3-I and LC3-II levels. **(b)** Quantification of LC3-II/LC3-I ratio / Actin. Values are expressed as mean \pm S.E.M., n=3. **(c)** Representative blot showing p62 protein levels. **(d)** Quantification of p62 / Actin. Values are expressed as mean \pm S.E.M., n=3.

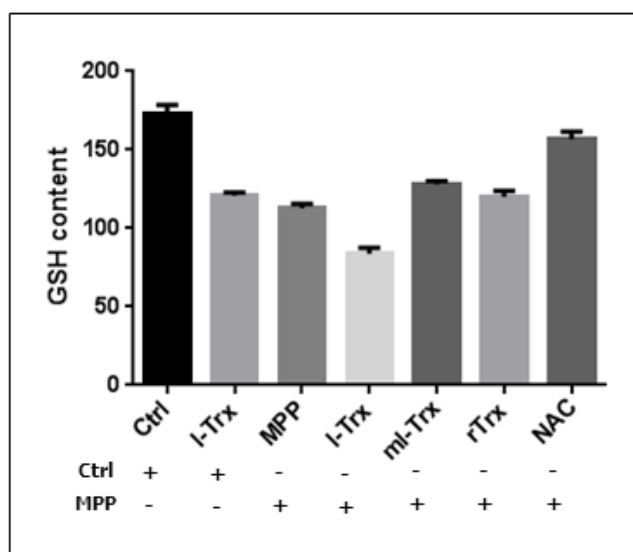


Figure 5: Effect of recombinant thioredoxin on GSH content in MPP⁺ treated SH-SY5Y cells
 Glutathione content (nmol/μg total protein) was assayed in SH-SY5Y cells treated for 24 hrs with 1mM MPP⁺ plus 2μM of recombinant protein or 2.5mM NAC. Values are expressed as mean ± S.E.M., n=4.

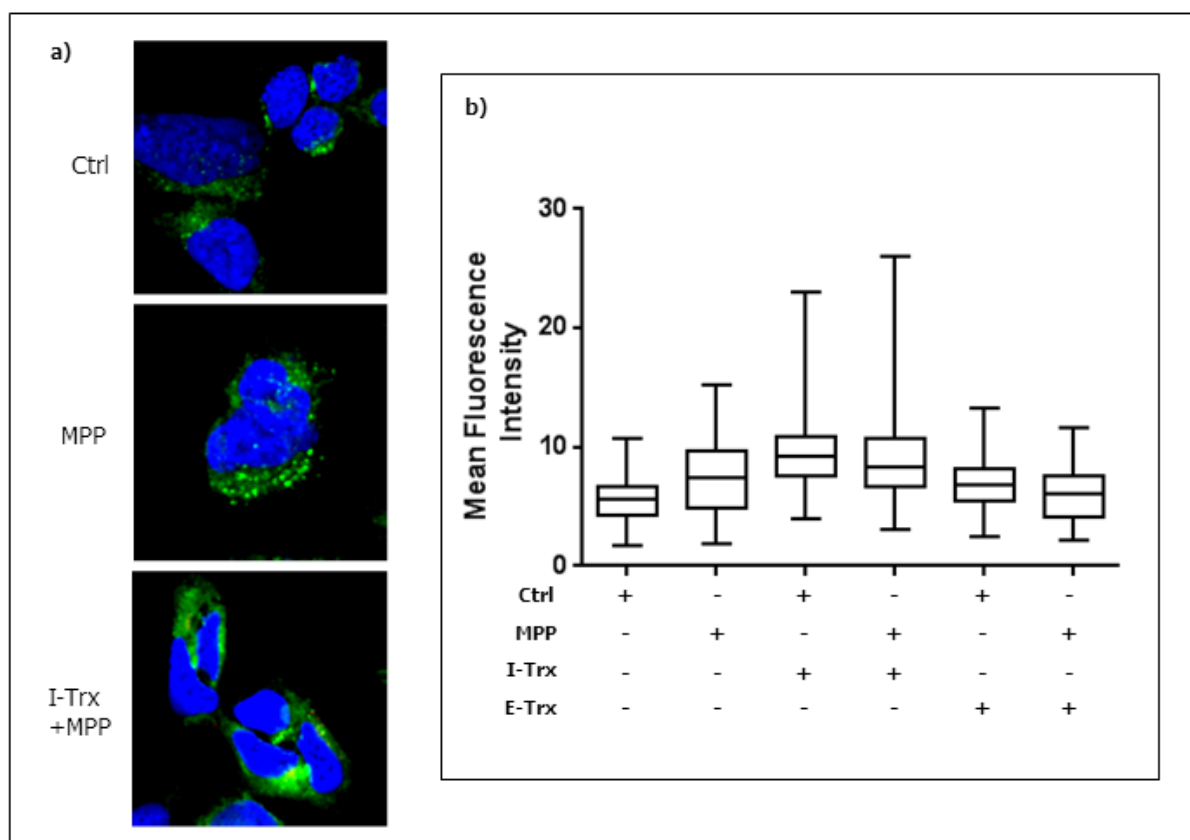
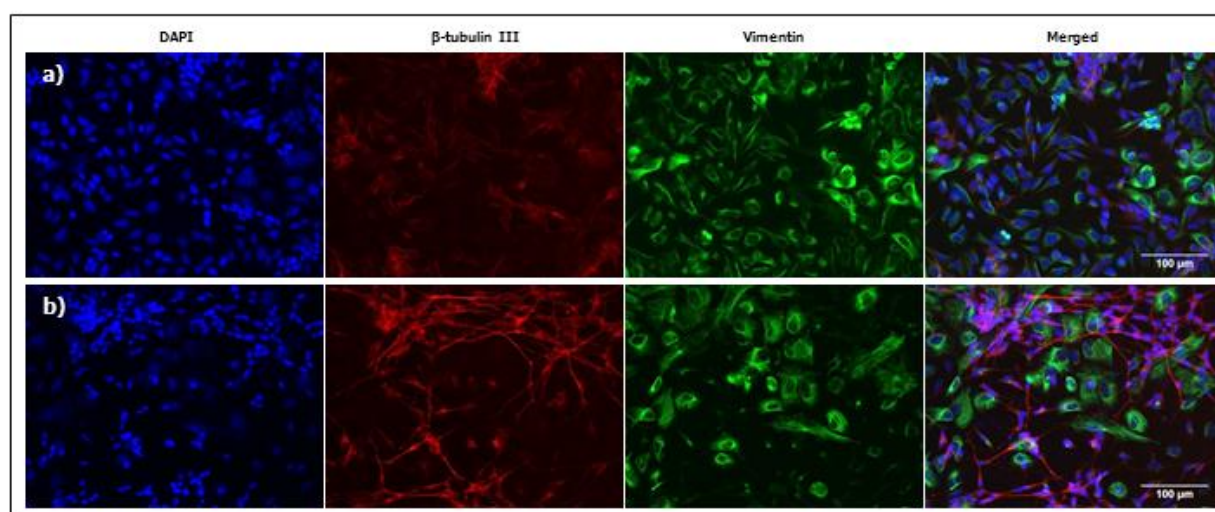


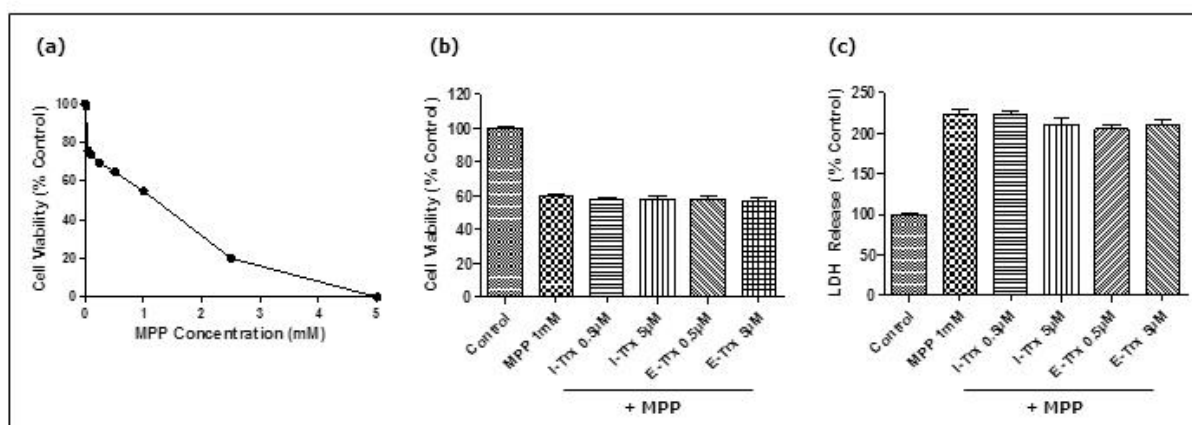
Figure 6: Effect of recombinant thioredoxin on GSH content in MPP⁺ treated SH-SY5Y cells
 (a) Representative images of CellROX Far Red stained SH-SY5Y cells treated for 6 hrs with 1mM MPP⁺ plus 2μM thioredoxin protein. (b) Quantification of ROS level. Mean fluorescence intensity of CellROX Far Red was measured and quantified using ImageJ. Values are expressed as box plots, n=4.



Supplemental figure 1: Retinoic acid differentiation validation of SH-SY5Y cells

SH-SY5Y cells differentiated for 7 days in retinoic plus 1% FBS media. Cells were immunostained for β -tubulin-III, a marker for neuronal cells, and vimentin, a marker for non-neuronal cells. The SH-SY5Y cell line consists of two sub-populations of cells; N-type cells which exhibit a neuroblastic phenotype and S-type cells which have an epithelial phenotype.

(a) In undifferentiated SH-SY5Y, N-type cells had short neurites and co-localization of β -tubulin-III and vimentin were observed. (b) In RA differentiated SH-SY5Y, marked neurite outgrowth was observed in the N-type cells with no co-localization of β -tubulin-III and vimentin. S-type cells were observed to have increased in size.



Supplemental figure 2: Effect of recombinant thioredoxin on cell viability and cell death in MPP treated differentiated SH-SY5Y cells

(a) SH-SY5Y cells were differentiated for 7 days in RA media, then treated with MPP at various concentration in RA media for 48 hrs to produce a cytotoxicity curve for MPP treatment. Cell viability was measured using MTT assay and reported as mean \pm S.E.M., $n=12$. (b) Cell viability in differentiated cells treated with 1mM MPP plus I-Trx and E-Trx for 48 hrs. Cell viability was measured using MTT assay and reported as mean \pm S.E.M., $n=12$. (c) Cell death was also measured with these treatment conditions using LDH assay and reported as mean \pm S.E.M., $n=6$.